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Phytotoxicity remediation in wheat (*Triticum aestivum* L.) cultivated in Cadmium- contaminated soil by intercropping design.

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Intercropping of suitable plant species has great value for the remediation of heavy metals-contaminated soils. The objective of the present study is to fix the problem of cadmium-contaminated soil by a fenugreek-wheat intercropping system. A pot experiment was managed to investigate the effect of wheat-fenugreek intercropping on growth, antioxidant activities and cadmium accumulation characteristics of tested plants grown under four cadmium concentrations (0, 5, 10 and 20 mg/kg soil). Intercropping of fenugreek with wheat significantly improved growth of both species as compared with monocropping plants. Meanwhile, the cadmium concentrations in the shoots and roots of wheat in the intercropping design were significantly ($p < 0.05$) lower than those in the monoculture. Additionally, fenugreek-wheat intercropping can improve plant resistance to heavy metals by decreasing the oxidative damage and increasing the antioxidant activities.

Keywords: Cadmium, Remediation, Intercropping, Fenugreek, Wheat, Antioxidants

INTRODUCTION

Soil contamination with heavy metals has become an important global challenge to environmental quality and human health. This situation has been worsened due to industrialization, urbanization, and excessive wastewater application for crop irrigation. Although some heavy metals, e.g. zinc, copper, and iron are considered plant micronutrients, there are others which are xenobiotic. Cadmium induces various noxious effects to different biochemical and physiological processes. Soils become contaminated with cadmium due to many natural and anthropogenic activities. (Hamid et al., 2019). In plants, Cd toxicity induces oxidative stress by increasing their active oxygen species and decreasing the content of antioxidant compounds. The oxidative damage is an etiological factor implicated in several chronic human diseases such as kidney stones, inflammation of glomeruli and tubular necrosis in the renal system (Hussain et al., 2019). Thus, remediating cadmium-contaminated soil has received a worldwide concern. The heavy metal remediation abilities of plant monocultures are often limited; therefore, the complementarity of co-planting species can be of significant value for the remediation of heavy metals found in the soil (Desjardins et al., 2017).

Intercropping is an efficient agronomic regime which can improve the effective utilization of soil nutrients, light, water, and space resources by crops, thereby increasing growth and yield of plants (Lu et al., 2017; Zhang et al., 2019). Intercropping system has the potential to remediate heavy metal-contaminated soils by regulating heavy metals absorption by plants, for saving safe crops (Zhang et al., 2019).Therefore, intercropping is a promising strategy and feasible practice of enhancing the remediation of heavy metals-contaminated soils.

Among cereals, wheat (*Triticum aestivum* L.), a potential source food for overgrowing world population, may accumulate above permissible limits of cadmium in its grains if cultivated on cadmium-contaminated soil (Hussain et al., 2019). Controlling Cd in the wheat aerial parts especially in grains is absolutely necessary to reduce Cd-mediated health risks. Fenugreek (*Trigonella foenum- graecum* L.) has multiple uses as food, feed and in traditional medicine as well as in improving the soil fertility through biological N_2fixation (Dadrasan et al., 2015; Salehi et al., 2018); therefore it provides a novel strategy for sustainable agricultural cropping systems (Salehi et al., 2018). Fenugreek is a powerful Cd hyperaccumulator (Zayneb et al., 2015), and can accumulate high concentrations of Cd in the root system (Abdel hameed and Metwally, 2019) to be utilized for remediation of cadmium-contaminated soil. No studies have focused on the remediation potential for Cd by fenugreek intercropped with wheat under induced Cd stress conditions. Therefore we try in this study to test remediation potential of fenugreek intercropped with wheat in cadmium-contaminated soil. This achieved through studying the effect of the intercropping on cadmium content in plants, oxidative stress markers and the antioxidant activities.

The hypothesis for the advantage of wheat-fenugreek intercropping in cadmium-contaminated soil is that the efficient Cd uptake ability of fenugreek, which decreases Cd availability in the rhizosphere of wheat, and hence Cd uptake by wheat root.

MATERIALS AND METHODS

Tested soil and plants

The present work was performed in the greenhouse of Botany Department, Faculty of Science, Zagazig University during winter 2017. The tested soil was collected from local agriculture soil at Sharkia Province. Soil' mechanical and chemical properties are mentioned in Table (1). The seeds of wheat and fenugreek were supplied by the Agriculture Research Centre, Ministry of Agriculture, Giza, Egypt.

Experimental design

Ten kilograms of soil was loaded into plastic pots (22.7 cm in diameter and 31 cm in height). Wheat and fenugreek seeds were sown in the pots after sterilization with 30% sodium hypochlorite solution for 2 minutes, washed four times with tap water, and rinsed in sterilized distilled water. Pots were classified into three groups: group1: monoculture of wheat(containing ten wheat seeds /pot), group2: intrercropping of fenugreek with wheat (five wheat seeds mixed with five fenugreek seeds /pot) and group 3:monoculture of fenugreek (ten fenugreek seeds /pot). The pots were irrigated weekly with tap water for 3 weeks, and uniform seedlings were selected for different treatments. For the monocultures, each pot contained four seedlings and for intercropping it contained two wheat seedlings and two fenugreek seedlings. Each group contained four levels of Cd: 0, 5, 10 and 20 mg/kg. Cadmium sulphate ($CdSO_4$) at different concentrations (0, 5,10 and 20 mg/l) were added to soil for one week before cultivation (Setkit et al., 2014). Different treatments (concentrations) were installed in three replicates for each treatment.

Morphological and biochemical analysis were performed at two growth stages: Late vegetative stage: after two months of cultivation and flowering stage: after three months of cultivation to evaluate the response of plants to cadmium stress during life cycle. After harvest time (after five months of cultivation), fenugreek and wheat plants were dried in an oven at 70°C for 48 hours until constant weight and dry weights were recorded. Dried plant and soil samples were used for estimation of cadmium content.

Measurement of growth indicators

Growth criteria measurements: Wheat and fenugreek plants were collected at vegetative and flowering stages for measurements of different growth criteria (Height of the plant, root length, fresh weight and dry weight).

Oxidative stress under Cd toxicity

1. Determination of malondialdehyde (MDA) content)

Plant malondialdehyde content (indicative of the degree of peroxidation of membranes under stress) was measured using the method described by Li (2000).

2. Determination of hydrogen peroxide (H_2O_2) content.

Hydrogen peroxide (H_2O_2) content was estimated as described by Alexieva et al., (2001). About 0.2 g of leaf samples of wheat and fenugreek were homogenized using 0.1% trichloro acetic acid

Table 1: Mechanical and chemical analyses of the experimental soil

pH	Humidity	Clay	Silt	Sand	Cd	organic matter content
8.24	5.23%	28.9 %	24.05 %	47.05 %	0.14 mg/kg	20.19 g/kg

(TCA). The homogenate was filtered through the Whatman No.1. filter paper. Then, 0.5 ml 100 mM K-phosphate buffer (pH 6.8) and 2 ml reagent (1 M KI w/v in fresh double-distilled water H_2O) were added to o.5ml of the leaf extract filtrate and then incubated in dark for one h. The absorbance of the developed color was measured at 390 nm against a reagent blank. The content of $H_2O_2$was calculated using a standard curve prepared with serial concentrations of H_2O_2.

2.5Antioxidant system

1. Phenolic compounds assay

The content of total phenolic compounds was assessed using the method of Strycharz and Shetty, (2002). Fresh leaves (1 g) were homogenized with 5 ml methanol. The homogenate was centrifuged at 10,000 rpm for 10 min and the supernatant was completed to a known total volume. Methanol extract was mixed with 1 ml of 95 % ethanol, 5 ml of distilled water and 0.5 ml of 50 % (V/V) Folin-Ciocalteu reagent; for the blank, 1 ml methanol replaced 1 ml extract. After 5 min, 1 ml of 5 % (w/v) Na_2CO_3 was added and mixed with samples. Tubes were covered and incubated in the dark for 10 min and the absorbance was measured at 725 nm. The total phenolic content was given based on the standard curve which was prepared using Gallic acid at concentrations of 2-20 µg/ml.

Flavonoid compounds assay

Total flavonoid content was determined according to the method of **Changet al., (2002)**.

Ascorbic acid (Vitamin C) assay.

Ascorbate was determined as the method given by Okamura (1981).

Analysis of heavy metal (Cd) in soil and plants

The different dried plants organs (shoot, root and seeds or grains) and soil sample were digested according to the method of Awofolu (2005). Cadmium content in different plants organs was determined by using acid digestion (HNO_3 and H_2SO_4), and the digested samples were analyzed by ICP-AES technique (inductively coupled plasma optical emission spectrometer). Cadmium concentration in different plant organs and soil were expressed as mg/g DW.

2.7.Statisticalanalysis

The obtained data were analyzed statistically paired sample T-test comparing between monoculture and intercropping. Differences were considered significant when $p<0.05$. All statistical analyses were carried out using the software SPSS, version 16 for windows.

RESULTS

Effects of intercropping design on plants growth under cadmium stress

Heavy metals have adverse effects on physiological and biochemical function of plants, most obvious effects are the inhibition of growth. Results in Table (2) indicate that cadmium with different concentrations had negative effect on wheat growth (root length, plant height, fresh weight and dry weight).The growth of the intercropped-wheat was significantly higher than monocropped-wheat under different levels of cadmium.

Effects of intercropping design on accumulation of cadmium in plants.

The Cd absorption characteristics of wheat and fenugreek plants were indicated through determination of cadmium content in soil, root, shoot and grains or seeds. Cadmium contents of wheat and fenugreek were significantly ($p<0.05$) differ between the monoculture and intercropping treatments as shown in Fig. (1). Cadmium concentration in the intercropped wheat were significantly ($p<0.05$) lower than that in monocultures .The content of cadmium in wheat plants was significantly ($p < 0.05$) decreased by intercropping with fenugreek plants. Contents of Cd in root, shoot and grains of intercropped-wheat were lower than those of monoculture- wheat under different concentrations of cadmium.

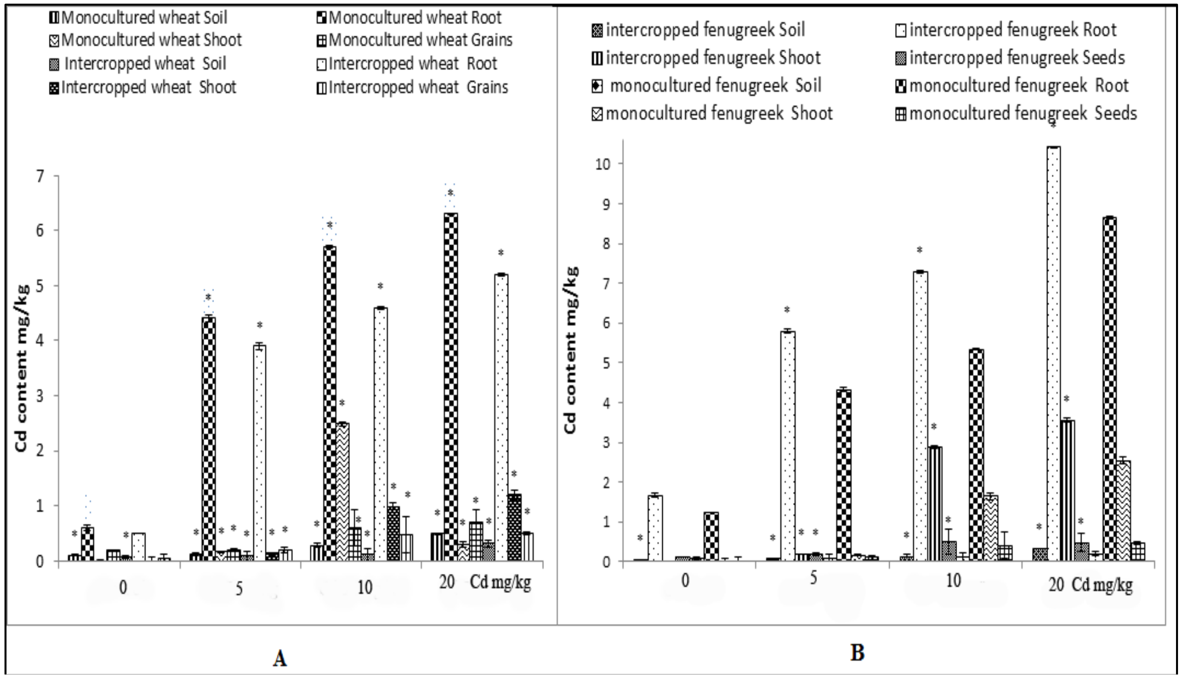

Figure 1 .Cadmium content (mg/kg) of wheat (A) and fenugreek (B) in monoculture and intercropping treatments.
Values are given as means of 3 replicates ± standard error. Within a column, means followed by asterisks are significantly different from mono-cultured treatments according to paired-samples *t* test at $p<0.05$.

Table (2): Effect of intercropping of wheat plants with fenugreek plants on fresh weight, Dry weight, shoot length, root length and leaf area of wheat and fenugreek plants under Cd stress.

Growth parameters		Shoot length (cm)		Root length (cm)		Plant fresh weight (g)		Plant dry weight (g)		Leaf area/leaf length $(cm^2)/(cm)$	
Treatments / Growth Q Stages		Vegetative Stage	Flowering Stage	Vegetative Stage	Flowering stage	Vegetative stage	Flowering Stage	Vegetative Stage	Flowering stage	Vegetative stage	Flowering Stage
Control	Wheat Monoculture	24	56	6.6*	7.3*	3.2*	10.9	1.8*	4.18 *	8.4*	30*
Cd 5mg/kg	Wheat Monoculture	20*	54	5.9	6.7	2.93	7.5	1.7	4.05	5.2*	23*
Cd10mg/kg	Wheat Monoculture	20*	54	4.4*	5.1*	1.25*	6.3	0.9*	3.81	6.1*	26*
Cd20mg/kg	Wheat Monoculture	17*	52	5.4*	5.9*	1.69*	4.9	1.1*	3.26 *	7.4	26*
Control	Wheat Intercropped	30.2 *	77*	8.4*	8.6*	4 *	12.5*	2.3	5.19*	18*	36*
Cd 5mg/kg	Wheat Intercropped	24 *	58 *	5.2*	6.2*	3 *	10.5	2	5	17.2	30
Cd10mg/kg	Wheat Intercropped	21.3*	58 *	6.1*	7.1*	2.05*	5.3*	1.6 *	3.8*	16.3 *	29*
Cd20mg/kg	Wheat Intercropped	23 *	49.9*	7.4*	7.9*	1.8*	9.25*	1.3*	4.5*	17	28
Control	Fenugreek monoculture	12*	30	6	7.6*	0.63*	2.9*	0.4*	1.35*	1.6*	2.2*
Cd 5mg/kg	Fenugreek monoculture	7.5*	29	6	6.9	0.44*	2.3	0.3	0.71*	1.4	1.8*
Cd10mg/kg	Fenugreek monoculture	6*	34	4*	5.7*	0.27*	1.15*	0.2*	1.29*	1.5	2
Cd20mg/kg	Fenugreek monoculture	6.5*	27*	4*	6.9	0.34*	1.5*	0.26*	0.65*	1.2*	1.7*
Control	Fenugreek intercropped	12.1*	31*	7.9*	11.3*	1.3*	3*	0.69*	1.45*	1.7*	2.3*
Cd 5mg/kg	Fenugreek intercropped	9.8*	28	6.5*	10.5	0.7*	1.29*	0.4*	1.3	1.4*	1.9*
Cd10mg/kg	Fenugreek intercropped	6.5*	35*	6*	11.1	0.4*	3.5*	0.24*	0.79*	1.5	1.8*
Cd20mg/kg	Fenugreek intercropped	7.8*	26*	5.5*	10.3*	0.61	1.01*	0.35*	0.92*	1.3*	1.8*

Values are given as means of 3 replicates. Within a column, means followed by asterisks are significantly different from the monocultured treatments according to paired-samples t test at $p<0.05$

Effects of intercropping design on oxidative damage induced by Cd.

Hydrogen peroxide content and lipid peroxidation were estimated to investigate the oxidative damage induced by cadmium. Lipid peroxidation was estimated by measuring malondialdehyde (MDA) content as shown in Fig. (2). Compared with the wheat monoculture plants, the MDA content was significantly decreased by the intercropping treatment under Cd stress. In comparison with the wheat monoculture plants, hydrogen peroxide content was significantly decreased in the intercropped wheat plants as shown in Fig. (2).

Antioxidative defense system under Cd stress.

Result in Fig. (3) showed that the intercropping system was significantly increased total phenolic contents in wheat plants as compared to the monoculture one. A more or less similar trend, which obtained in phenolic contents, is present in flavonoids contents in either mono or intercropped wheat plants as shown in Fig. (3).As shown in Fig. (3), Application of intercropping design was significantly increased the ascorbic acid content in wheat plants. The maximum content is 1.82 mg/mL recorded in Cd10 mg/kg treatment in intercropped wheat plants at flowering stage

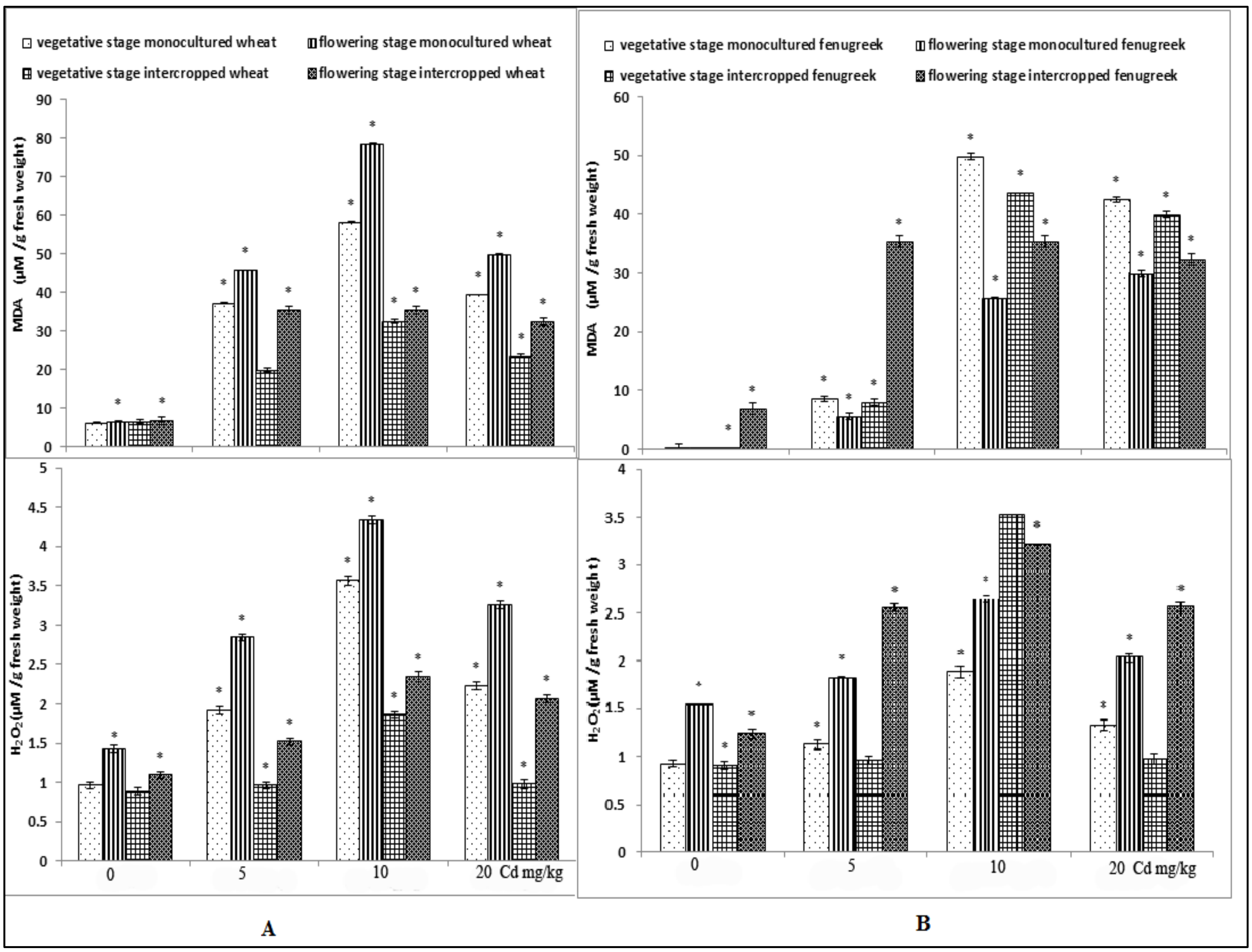

Figure 2; Oxidative damage markers (MDA content (µM /g fresh weight) and H_2O_2 (µM /g fresh weight))in wheat (A) and fenugreek (B) in monoculture and intercropping treatments.

Values are given as means of 3 replicates ± standard error. Within a column, means followed by asterisks are significantly different from the mono cultured treatments according to paired-samples *t* test at p<0.05.

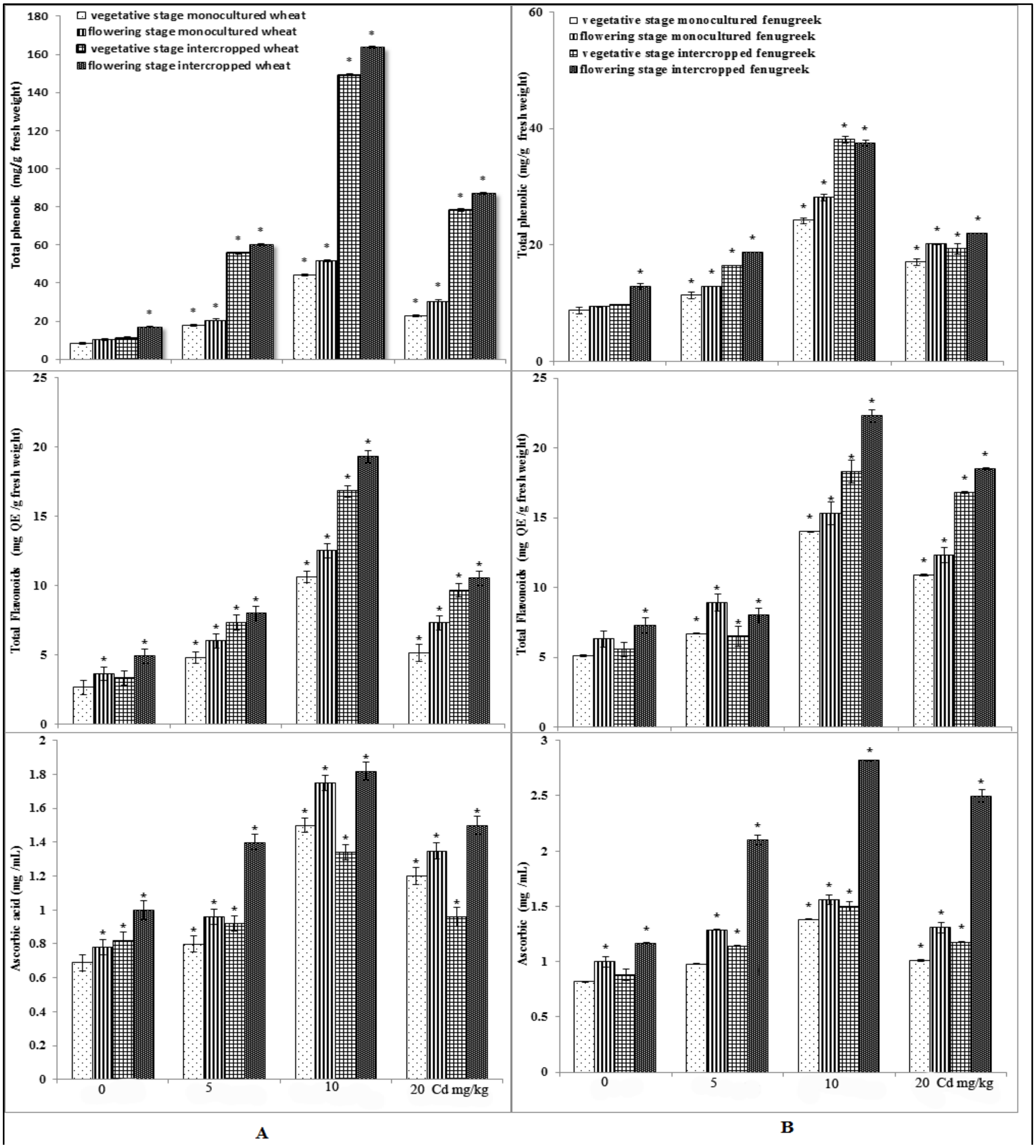

Figure.3.Non-enzymatic antioxidants content (Total phenolic (mg /g fresh weight), Total Flavonoids (mg QE /g fresh weight), Ascorbic acid content (mg /mL)) in wheat (A) and fenugreek (B) in monoculture and intercropping treatments.

Values are given as means of 3 replicates ± standard error. Within a column, means followed by asterisks are significantly different from the mono-cultured treatments according to paired-samples *t* test at $p<0.05$.

DISCUSSION

Intercropping design in which two crops are cultivated in the same area and at the same time is able to enhance crop diversity at the field scale and maintain ecosystem functions by increasing nutrient bioavailability in plant shoots and improving ecological function (Zu et al., 2017; Tian et al., 2019). Our result showed that growth criteria of the intercropped plants both wheat and fenugreek were significantly enhanced ($P < 0.05$) compared with monocropping plants. Intercropping with legumes enhance soil fertility by increasing soil nitrogen (N) through the N-fixing ability of rhizobacteria, thereby allowing more fixed-N to remain in the upper soil layers and become available for plants (Chapagain and Riseman, 2014). Therefore, a fenugreek-wheat intercropping system could be a good strategy for alleviation of Cd toxicity on plants growth in Cd-contaminated soil.

Intercropping design can decrease the quantity of trace metal in the intercropped crops,improve the remediation opportunities of the enrichment plants on polluted soils and reducing the food safety risks (Huang et al., 2006; Zu et al ., 2017). In this study, Cd concentrations in shoots and grains of wheat were significantly decreased by intercropping with fenugreek under all cadmium levels as compared to the monoculture plants. Similarity, concentrations of Cd decreased in the shoots of tamarillo by intercropping of tamarillo with American black nightshade (*Solanum photeinocarpum* Mill.) as compared to the monoculture plants under cadmium stress (Lin et al., 2018). Agroecology, other studies reported that intercropping and inter-planting designs, depends on the principle of allelopathy, could improve agricultural productivity by improving the yield and quality of crops (Liu et al., 2019). The allelopathic potentional of wheat during the intercropping design improves the cultivated soil quality by adding nutrients for crop plants, reducing the weeds and pests infestation and conferring tolerance against environmental stresses (Aslam et al., 2017). Hence, intercropping of fenugreek with wheat not only improved wheat growth alone but also improved fenugreek growth.Intercropping system can affect the exchangeable levels of Cd in the soils (Murakami et al., 2008). Thus, fenugreek-wheat intercropping system would change and affect the uptake of cadmiumby plants. The nitrogen fixation at the root of fenugreek can provide nutrients to wheat to enhance the tolerance of wheat to Cd as nitrogen is an important detoxification factor for cadmium stress (Zhang et al., 2014; Cui et al., 2018). Therefore intercropping of fenugreek with wheat would be a good approach for remediation of Cd and improve the food quality of wheat in Cd-contaminated soils.

Malondialdehyde (MDA) -a lipid peroxidation product- is correlated with the increase in toxicity of heavy metals (Kumar et al., 2019), so the extent of peroxidation of lipids in the Cd-treated plants were determined through MDA assay. Cadmium caused cellular damage in plants, which can be examined by estimating the lipid peroxidation level in plants as by increasing the content of MDA, the degree of peroxidation of the cell membrane was increased under Cd stress (Tammam et al., 2016; Rizwan et al., 2019). Also, malondialdehyde (MDA) contents in shoots and roots of wheat plants were increased under cadmium stress (Chen et al., 2014; Rizwan et al., 2016). Peroxidation of lipids results in breakdown of plant tissues, which in turn decreased the growth of plants (Pandey, 2018).

Hydrogen peroxide has important roles in some physiological processes such as cell elongation, growth, development and protection of plants from severe environmental stresses (Petrov and Van Breusegem , 2012). Excessive heavy metals increase the H_2O_2 level and lipid peroxidation in plants (Cui et al., 2018). In our study, compared with the monoculture treatments, intercropping significantly decreased MDA and H_2O_2 contents in both wheat and fenugreek. The results indicated that intercropping treatment can mitigate the toxic effects of heavy metals by decreasing the harmful substance and alleviating oxidative damage.

Plants possess an efficient anti-oxidative defense system to detoxify the ROS and to be prevented from oxidative damage (Rehman et al., 2019). Non enzymatic anti-oxidants like phenolics, play an important role in plant defense from most ion toxicities (Singh et al., 2016). Phenolics are able to repair or prevent injuries caused by stress by interaction with many cell structures and biochemical pathways (Amari et al., 2017). Phenolics have an important role in Cd detoxification due to their high reactivity as hydrogen or electron donors, stabilize and delocalize the unpaired electron (chain-breaking function) (Huang et al., 2005). Increasing in phenolics contents may be a cellular adaptive mechanism for scavenging oxygen free radicals during stress (Mohamed and Aly, 2008). Some flavonoids have the ability to protect plants from heavy metal stress by chelating transition metals that generate hydroxyl radical through the Fenton's reaction to produce complexes that save the metal ions from their participation in free-radical generation (Michalak, 2006). It is important to evaluate changes of flavonoids and phenols contents under heavy metal stress. This is due to the important that heavy metals could affect each of them at different content creating variations in some parts of plants physiology and not in others to access an adequately high flavonoids and phenols amounts of plants applied in the process is the basis of induced remediation efficiency. Ascorbic acid (AsA), one of the most powerful antioxidant in plants, is believed to protect plants from oxidative damage by scavenging free oxygen radicals. An increase in the level of AsA in plants is one of the approaches of attaining stress tolerance (Aziz et al., 2017). Ascorbic acid could improve the tolerance of wheat seedlings to cadmium stress (Wang et al., 2017)and this could implicate its important role as an antioxidant acting as part of the plant defense system. Our study indicated that the intercropping of fenugreek with wheat plants resulted in significantly increasing in phenolic, flavonoids and ascorbic acid contents as compared to monoculture plants. Analysis of antioxidants revealed that the intercropping system of fenugreek with wheat played an important role in protecting plants against Cd-induced oxidative stress.

CONCLUSION

In conclusions, the intercropping of fenugreek with wheat can effectively reduce the adverse impacts of cadmium on plant growth in Cd-contaminated soils. The growth of wheat was significantly increased by intercropping with fenugreek. Additionally, fenugreek-wheat intercropping system can increase the antioxidant defense mechanism and decrease the oxidative damage by Cd to enhance the resistance of plants in Cd-contaminated soil.

CONFLICT OF INTEREST

The authors declared that present study was performed in absence of any conflict of interest.

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AUTHOR CONTRIBUTIONS

All authors contributed equally in all parts of this study**.**

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